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AN INCREASE IN PROTEIN SYNTHESIS DURING RIPENING OF THE BANANA FRUIT

C. J. BRADY, JAMES K. PALMER,* P. B. H. O'CONNELL and ROBERT M. SMILLIE

Plant Physiology Unit, C.S.I.R.O. Division of Food Preservation, Ryde, and School of Biological Sciences, The University of Sydney, Sydney, N.S.W. 2006, Australia

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Abstract—Protein synthesis in ripening banana fruit slices was investigated. Measurements were made of the rate of incorporation of amino acids in a clearly defined protein fraction obtained by molecular sieve chromatography of phenol/acetic acid/water extracts of pulp tissue. During the early part of the climacteric rise which followed ethylene treatment, both the rate of uptake of amino acids into cells, and the rate of incorporation into protein increased. An involvement of re-directed protein synthesis in ripening was also indicated by (1) changes in the patterns of proteins observed after gel electrophoresis, and (2) the inhibition of ripening by specific inhibitors of protein synthesis. These results do not support previous reports of a progressive decline throughout ripening in the rate of protein synthesis in the banana fruit.

INTRODUCTION

THE RIPENING of fruits involves a dramatic increase in the activity of a number of enzymes. Thus in the ripening fruit marked changes in texture, sweetness, flavour and colour occur. Evidence of an increase in the rate of protein synthesis,[1,2] in the protein content,[3-6] and of a changed pattern of nucleic acid synthesis[7] early in the ripening phase may indicate that at least some of these changes are controlled at the level of transcription or translation of the genome. Frenkel *et al.*[8] have concluded from studies on pome fruits that ripening involves directed synthesis of new enzymes required for ripening.

There is evidence that in banana fruit-pulp tissue, the rate of protein synthesis declines progressively from the initiation of the climacteric period.[9] The decline in protein synthesis parallels an increase in the proportion of cells in tissue slices whose contents leak freely into a bathing solution.[9] This evidence suggests that ripening in banana results primarily from a decline in intracellular organization, and the consequent action of enzyme systems pre-existing in the cells.

In the work presented here, we have re-examined the role of protein synthesis in banana ripening. Features of this study were the use of 6–8 mm thick fruit slices,[10] to limit the effects

* Present address: Dept. of Nutrition and Food Science, M.I.T., Cambridge, Mass., U.S.A. 02139.

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of slicing,[11, 12] and the use of phenol extraction, followed by molecular sieve chromatography to achieve a rigorous separation of protein from non-protein constituents.[13–15]

RESULTS

1. *Separation of Protein from Non-Protein Compounds*

For measurements of the rate of protein synthesis, a complete separation of protein from non-protein precursors is essential. This was accomplished by successive extraction of pulp tissue with aqueous TCA (5 per cent, w/v), chloroform/ethanol (2/1, v/v) and phenol/acetic acid/water (PAW) (1/1/1, w/v/v)[15] followed by molecular sieve chromatography of the PAW extracts.[14] The amount of nitrogen in typical extracts prepared from pre-climacteric and climacteric samples of pulp tissue is shown in Table 1. The bulk of the nitrogen in PAW extracts was of large molecular size (Fig. 1); free amino acids were found to account for less than 5 per cent of the ninhydrin colour yield of the peak eluted after the protein. PAW extracts contain, then, no more than 0·2 per cent of the free amino acids of the original tissue, and these were well-separated from the protein on Sephadex columns.

TABLE 1. NITROGEN IN FRACTIONS OF BANANA-PULP TISSUE

	Maturity	
	Pre-climacteric	Climacteric
Respiration*	28–30	72–80
Dry matter, per cent fresh wt.	27·0	25·9
Total N, per cent dry matter	0·89	0·95
TCA-soluble N†	40·6	39·6
$CHCl_3$–EtOH soluble N†	6·5	9·2
$CHCl_3$–EtOH soluble after washing†‡	0·6	0·8
PAW-soluble N	40·5	40·1
N in residue†	12·5	16·1
Recovery of N (%)	100·1	105·0

* Determined on samples of the whole fruit immediately prior to analysis and expressed as mg CO_2/kg fresh wt./hr.

† As percentage of N in the pulp prior to fractionation.

‡ N remaining in solution in the $CHCl_3$-rich phase after repeated partitioning against 0·9 N NaCl.

2. *The Uptake and Incorporation of Amino Acids*

Planning and interpretation of ^{14}C incorporation experiments required an estimate of the free amino acid content of banana-pulp tissue. Published values[16, 17] differ widely from each other. Table 2 shows the free amino acid content of pulp from green and ripening

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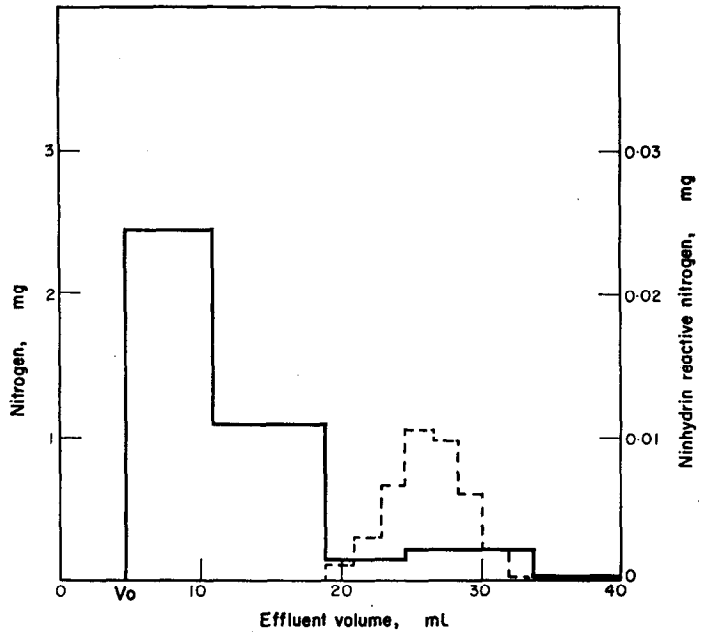

FIG. 1. MOLECULAR SIEVE CHROMATOGRAPHY OF PAW EXTRACT OF CLIMACTERIC PULP SAMPLE. 2·0 ml of extract (4 mg/ml total N) was loaded onto a column of Sephadex G-75 in PAW. The column was 10 × 2 cm with a void volume of 5·0 ml (determined using horse-heart cytochrome c). Amino N (dotted line) was estimated on individual trailing fractions by the ninhydrin method, and total N (solid line) on bulked fractions by the Kjeldahl method. The amino N is expressed on a scale 100 × that of the total N.

TABLE 2. AMINO ACIDS AND AMIDES IN TCA EXTRACTS OF BANANA PULP

	μmoles/g fresh wt. of pulp		
	Pre-climacteric		
	I	II	Climacteric
Aspartic acid	2·44	2·61	3·70
Asparagine	3·33	4·52	3·99
Threonine	0·36	0·38	0·86
Serine	0·55	0·58	0·62
Glutamic acid	1·35	0·43	0·85
Glutamine	4·27	3·48	2·00
Proline	0·18	0·18	0·17
Glycine	0·55	0·55	0·65
Alanine	0·56	0·54	0·15
Valine	0·11	0·16	0·18
Iso-leucine	0·12	0·12	0·15
Leucine	0·20	0·21	0·31
Tyrosine	0·07	0·10	0·07
Phenylalanine	0·10	0·13	0·10
γ-Aminobutyric acid	0·55	0·41	1·25
Lysine	1·07	1·22	1·40
Ornithine	0·05	0·05	0·04
Histidine	6·09	7·53	9·08
Arginine	1·25	1·16	1·08

Samples were from different batches of fruit.

bananas. These concentrations are 10–100 times higher than those calculated from the results of Steward *et al.*,[16] although the total nitrogen content of our samples was only slightly higher. Lysine was chosen for initial incorporation experiments. The rate of penetration of lysine into cells of the central pulp zone of slices was estimated by measuring the amounts of TCA-soluble ^{14}C and amino nitrogen which diffused from thin slices of the pulp into water. The excess percentage of ^{14}C over amino nitrogen diffusing was taken to represent ^{14}C-lysine outside the cell membranes. Penetration of lysine into pulp cells was found to be rapid (Tables 3 and 4).

TABLE 3. THE RATE OF UPTAKE OF INFILTRATED ^{14}C-LYSINE INTO PULP CELLS

	Time from infiltration (min)			
	0	30	90	150
A. Amino N diffusing (%)	24·4	26·2	31·9	29·1
B. ^{14}C diffusing (%)	92·5	66·4	51·1	57·2
C. TCA-soluble ^{14}C within cells (%)	10·0	45·5	71·9	60·5
D. Total uptake (%)	11·3	49·8	81·8	85·0

Aged, pre-climacteric slices were vacuum infiltrated (2 min at 26 in. of mercury) with ^{14}C-lysine (0·23 μc and 27·4 mμmoles/ml) and chloramphenicol (50 μg/ml). Slices (ten per time interval) were incubated in the dark at 25°. At appropriate times, amino N and ^{14}C diffusing into cold aerated distilled water were measured as described under Methods. *A*, *B*, and *C* are expressed as the percentages of amino nitrogen or ^{14}C in the diffusates plus extracts. *C* was derived from *A* and *B*, as $C = (100 - B) + (100 - B) \times [A/(100 - A)°]$. Total uptake (*D*) included as well ^{14}C in PAW extracts, and is expressed as a percentage of total ^{14}C recovered.

TABLE 4. EFFECT OF ETHYLENE TREATMENT ON THE UPTAKE AND INCORPORATION OF L-^{14}C-LYSINE BY PULP CELLS

	Time of exposure to ethylene (hr)		
	0	13	40
A. Amino N diffusing (%)	16·1	15·9	36·3
D. Uptake (dpm × 10^{-3}/g)	45·9	60·8	44·5
Uptake (%)	72·3	90·6	67·6
Incorporation			
dpm × 10^{-3}/mg protein	1·26	2·68	0·16
dpm × 10^{-3}/μmole protein lysine	2·73	4·07	0·19
Percent of uptake	23·6	35·6	8·3

^{14}C-lysine (0·3 μc and 1·6 mμmole/ml) and chloramphenicol (50 μg/ml) was vacuum infiltrated (26 in. of mercury for 2 min) into slices prepared 5 days previously. Prior to infiltration one group of slices was exposed to 60 ppm of ethylene for 13 hr, and a second group for 16 hr followed by 24 hr in air. Each treatment included ten slices drawn from five bananas all from one hand. After infiltration, each slice was incubated for 60 min at 25° in the dark, and the central pulp zones recovered and analysed. Uptake was measured as described under Methods, and is expressed as a percentage of total ^{14}C recovered. *A* is a measure of cell leakage and is expressed as a percentage of the TCA-soluble amino N in the system.

Lysine was incorporated into protein in pre-climacteric tissue at an approximately constant rate for about 90 min after infiltration; in experiments comparing different tissues, incubation times of 60 min or less were used. All protein preparations were hydrolysed, and examined by two-dimensional paper chromatography and radioautography. No radioactive compounds, other than ^{14}C-lysine, were detected.

Both the rate of uptake of lysine into cells and the rate of incorporation of lysine into protein increased in pulp tissue following treatment of slices with ethylene for a short time (Table 4). Since incorporation increased relative to uptake, the rate of protein synthesis in the tissue increased. The decline in incorporation in the 40 hr, late climacteric tissue was associated with an increase of leakage of amino acids, and may in part reflect increased dilution of isotope by endogenous amino acid.

TABLE 5. EFFECT OF ETHYLENE TREATMENT ON THE UPTAKE AND INCORPORATION OF L-^{14}C-VALINE BY PULP CELLS

	Time of exposure to ethylene (hr)		
	0	13	25
Respiration (mg CO_2 kg^{-1} hr^{-1})	16–22	36–45	50–58
A. Amino N diffusing (%)	24·9	27·1	35·6
D. Uptake (dpm × 10^{-3}/g)	13·8	19·9	16·0
Uptake (%)	73·8	91·2	89·8
Incorporation			
dpm/mg protein	971	1607	1577
dpm × 10^{-3}/μmole protein valine	1·95	4·03	4·68
Percent of uptake	22·4	40·9	29·0

For each treatment, sixteen slices were aged for 4 days after slicing and then, when appropriate, treated in a flowing air stream with 10 to 15 ppm of ethylene for the times indicated. Each slice was infiltrated (26 in. of mercury for 1 min) with a solution containing L-^{14}C-valine (0·21 μc and 0·8 mμmole/ml) and chloramphenicol (50 μg/ml). After incubating in the dark at 25° for 45 min, the central pulp zones were recovered and analysed. Uptake was measured as described under Methods, and is expressed as a percentage of total ^{14}C recovered. *A* is a measure of cell leakage, and is expressed as a percentage of the TCA-soluble amino N in the system.

When valine was used as precursor (Table 5) an increase both in uptake and in incorporation rate was observed in ethylene-treated slices. Incorporation increased more than did uptake. A comparison of samples 13 hr or 25 hr after exposure to ethylene showed little difference in incorporation rate although the amount of leakage of amino acid from the tissue to water had increased sharply in the interval. Infiltrated ^{14}C-valine contributed no label to neutral or acidic compounds, nor to other amino acids, during 60 min incubation of pre-climacteric slices, nor during the treatments detailed in Table 5.

3. *Effect of Inhibitors of Protein Synthesis on Ripening*

Cycloheximide and *p*-fluorophenylalanine inhibited the ripening of banana slices subsequently treated with ethylene (Fig. 2). Inhibition by cycloheximide was apparent with concentrations down to 0·25 μg/ml. Above 2 μg/ml ripening was severely inhibited, but necrotic zones appeared indicating cellular damage. L-Phenylalanine (1 mole/mole) reversed the inhibition by *p*-fluorophenylalanine (1000 μg/ml).

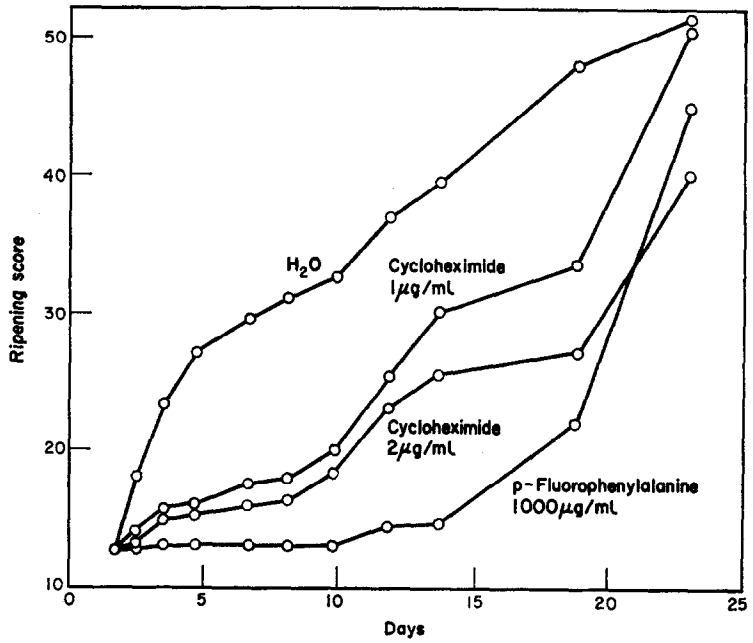

FIG. 2. INHIBITION BY CYCLOHEXIMIDE AND *p*-FLUOROPHENYLALANINE OF RIPENING RESPONSE TO EXOGENOUS ETHYLENE.

Slices were infiltrated (1 min, 12 in, mercury) at time 0, immediately exposed to ethylene (60 ppm) for 12 hr in a large sealed container and then incubated at 23° in circulating moist air. See Experimental for method of scoring ripening.

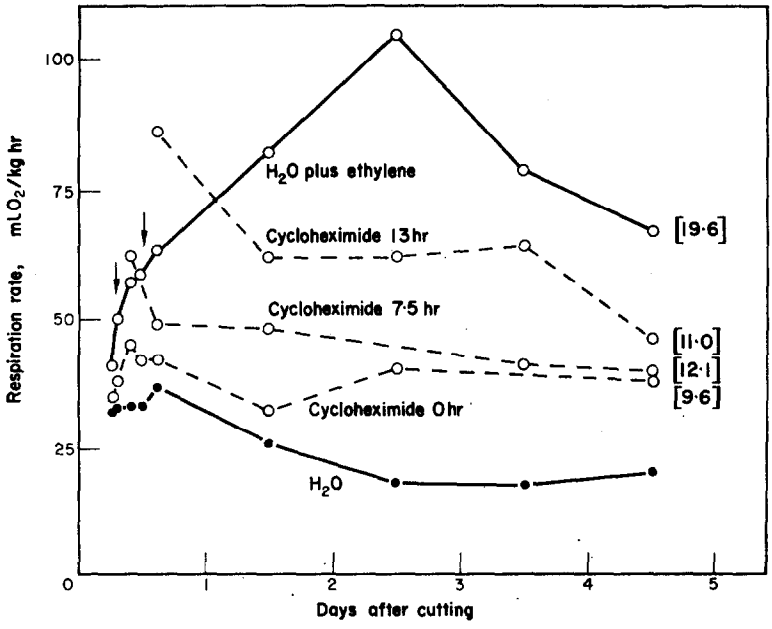

FIG. 3. THE EFFECT OF CYCLOHEXAMIDE ON THE RESPIRATORY PATTERN OF BANANA SLICES VENTILATED CONTINUOUSLY WITH 10–15 ppm OF ETHYLENE.

Slices were infiltrated (1 min, 16 in. mercury) with water or cycloheximide solution (2 μg/ml) immediately after cutting, or 7·5 or 13 hr later, as indicated by arrows in the fig. The bottom trace shows the respiration rate of slices not treated with ethylene. Figures in brackets show the percentage content of soluble solids at the completion of the experiment.

Infiltration of cycloheximide (1–2 μg/ml) caused no change in the respiration rate of unripe slices during the ensuing 3 days. However, infiltration of cycloheximide into slices during the climacteric induced by added ethylene (Fig. 3) both prevented any further rise in respiration and limited starch hydrolysis as estimated by the increase in soluble solids. In ripening tissue, cycloheximide did not inhibit the immediate rise in respiration which results from infiltration, nor cause a rapid decline in respiration towards the pre-climacteric level.

Puromycin (100 μg/ml) also inhibited ripening, but chloramphenicol (50–2000 μg/ml) consistently had no influence on ripening. Azetidine-2-carboxylic acid (100 μg/ml) caused a premature de-greening of the peel accompanied by a striking development of latent micro-organisms.

4. *The Soluble Pulp Proteins*

If the increased protein synthesis which occurs in the early climacteric is associated with the production of proteins specifically involved in ripening, then changes may be detected in the types of proteins in pulp extracts. Accordingly, extracts of frozen pulp tissue, taken from fruit of six maturities from pre-climacteric to yellow ripe, were examined by polyacrylamide gel electrophoresis.

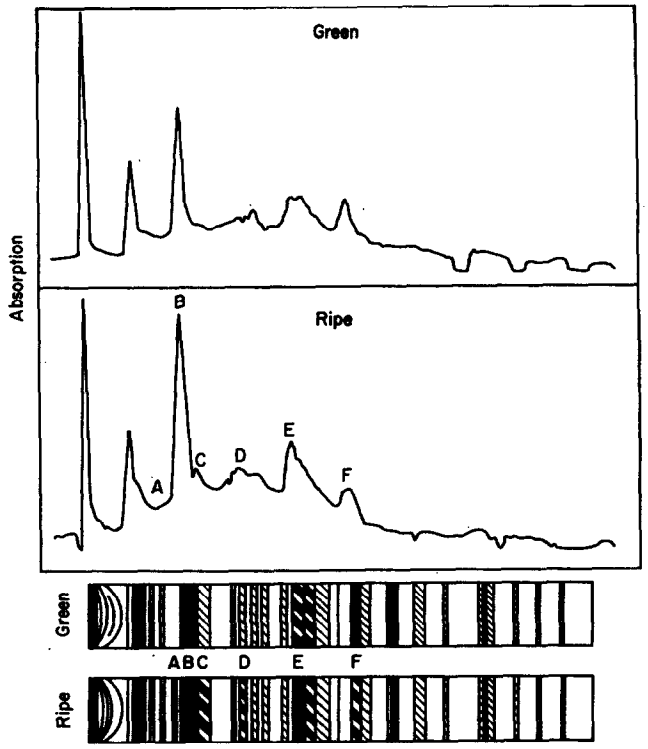

FIG. 4. SOLUBLE PROTEINS OF THE PULP TISSUE OF FRUIT AT SIX STAGES OF RIPENING WERE EXAMINED IN 7·5 PER CENT POLYACRYLAMIDE GEL USING AN ANIONIC (pH 8·3) SYSTEM, WITH A CURRENT OF 3 mA/TUBE.

Direction of travel is from left to right. Loadings used were 170 μg protein/tube. The comparisons presented are of pre-climacteric (green) and peak climacteric (ripe) tissue. The relative increases at *A*, *B*, *C*, *D* and *E* and the decrease at *F* developed progressively through the climacteric.

With proteins moving as anions (initial pH 8·3) better resolution was obtained with Tris–borate–EDTA than with Tris–glycine buffers. When evaluated at constant protein load per gel, differences in the protein pattern were observed to develop as the tissue moved from the pre-climacteric to peak climacteric condition. These progressive changes were recorded as densitometer traces, and have been confirmed with a number of batches of fruit. A comparison of pre- and peak-climacteric samples is presented in Fig. 4.

Differences were also apparent in zones of high R_f with proteins moving as cations in a low pH (4·5) electrophoretic system. Satisfactory resolution of the proteins of post-climacteric fruit was not obtained.

DISCUSSION

The relatively low protein content of bananas coupled with a high concentration of polysaccharide and the presence of reactive phenolic substances complicates the study of protein metabolism during ripening.[18–20] These polysaccharides and phenols change in amount and in type during ripening. Such factors make difficult the distinction between the occurrence and the recovery of particular proteins in unaltered form,[21,22] and influence the certainty with which isotope incorporation into a "protein" fraction can be taken as a measure of protein synthesis. In this latter regard, the selective solution of proteins from carbohydrate components, and the possibility of freeing proteins of lower molecular weight contaminants,[14,15] is a distinct advance in technique. PAW dissolved about 70 per cent of the protein of banana pulp and if the tissue was first extracted with 5 per cent TCA the PAW extracts were virtually free of carbohydrate.

The elution pattern of nitrogen when the PAW extracts were examined by molecular sieve chromatography shows that aqueous TCA efficiently extracted low-molecular-weight nitrogenous compounds, including free amino acids, while leaving undissolved some compounds of intermediate molecular size. We have investigated neither the chemistry nor the metabolism of this intermediate group, but have separated them from the "protein" fraction before measuring incorporation into the latter. Likewise, we have not measured incorporation into the nitrogenous compounds, presumably including wall proteins, left in the residue after PAW extraction.

Our conclusions concerning increased protein synthesis during the early climacteric are in substantial agreement with those of Richmond and Biale[1,2] with avocado and Frenkel *et al.*[8] with pears, but differ from those of Sacher[9] who studied banana-pulp slices. Sacher interpreted his results as meaning a decline in incorporation from pre-climacteric through to climacteric fruit; this decline was associated with increased free space in the tissue, and thus increased dilution of exogenous by endogenous amino acid. To demonstrate this dilution, he measured incorporation at a range of concentrations of external amino acid. A comparison of these concentrations (0·12–5·0 mM for leucine; 1·0–50·0 mM for phenylalanine) with the amino acid content of banana pulp we have observed (Table 2), indicate that the concentrations chosen were too high to sensitively measure dilution effects.

Because of the uncertainties surrounding the quantitative interpretation of incorporation studies, whether thin slices or infiltrated sections are used, we have not attempted to define

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fully the influence of endogenous dilution on the observed passage of amino acid into protein. Other studies[23,24] have shown that permeability barriers decline during the climacteric. Endogenous dilution is, therefore, likely to increase. Our results can then only be interpreted as indicating an increased rate of protein synthesis as ripening commences, and certainly not a progressive decline.[9]

Labelled amino acids infiltrated into the intercellular spaces will be rapidly diluted by that portion of the amino acid pool which diffuses readily from tissue slices. For pre-climacteric fruit, this portion is about one-fifth of the tissue amino acids. Calculating the specific activity of amino acid entering the protein as that of the introduced amino acid diluted with one-fifth of the endogenous pool, we have measured mean turnover rates in the range 0·10–0·35 per cent per hr. for the protein of pre-climacteric pulp cells. If the introduced amino acid is further diluted by that portion of the amino acid pool which does not diffuse from tissue slices, these rates will be correspondingly higher. An increase in the synthetic rate in the early climacteric represents an addition to an already appreciable activity.

Infiltration, like slicing,[25] causes tissue respiration to rise.[10] Thus incorporation is measured during a period of enhanced respiration. Incubation was for an hour or less after infiltration. Lag periods from two to several hours before measurable increase in enzyme activity in response to slicing[26,27] or to infiltration,[28] have been reported. Studies on amino acid incorporation and sensitivity to specific inhibitors[27–30] indicate a lag of about 2 hr before the rate of protein synthesis increases in response to slicing. From this it appears to follow that if infiltration induces typical ageing responses, they would not have developed sufficiently during the incubation times used to materially influence the results.

While the inhibition of ripening by cycloheximide, *p*-fluorophenylalanine and puromycin is consistent with a need for continued protein synthesis if ripening is to proceed, such studies cannot establish that new types of proteins need to be synthesized. The appreciable turnover-rate shown by pre-climacteric tissue may need to be maintained, if the drastic re-orientation of metabolism which occurs during ripening is to be accomplished.

Frenkel *et al.*[8] reported cycloheximide to inhibit characteristic ripening changes in pome fruits, without influencing the climacteric. They interpreted this to mean that the climacteric rise is only co-incidental to ripening. In bananas, cycloheximide inhibits the development of the respiratory changes, but does not appear to effect respiration itself. Whether the arrest of the climacteric reflects an effect on protein synthesis, or some other effect of cycloheximide, e.g. on energy metabolism,[31] is not known.

The ultimate objective of our electrophoretic studies of the pulp proteins is to measure the incorporation of ^{14}C amino acids into specific proteins at various stages of ripening and then to identify any proteins which are preferentially labelled during the climacteric. These studies are continuing, but the gel separations themselves already indicate progressive changes in banana proteins during the climacteric. Frenkel *et al.* have reported increased synthesis of specific proteins during ripening of pome fruits.

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Our studies on protein synthesis, with inhibitors and on the changes in pulp proteins, support the conclusion that the initial stages of banana ripening involves a re-orientation of protein synthesis. Sequential changes in cell permeability and in compartmentation undoubtedly play a role, particularly in the later stages, but do not appear to be the primary events in ethylene-induced ripening.

EXPERIMENTAL

Source of Fruit

Bananas of the Williams hybrid strain of the Dwarf Cavendish variety, slightly immature by commercial standards, were obtained from Avoca, N.S.W. Fruit, within 24 hr of harvest, was equilibrated in a flowing air stream and the respiration rate was measured. Fruit, whose respiration rate remained constant for 2–3 days, was selected as experimental material.

Nitrogen Distribution

Total N was estimated by the Kjeldahl method with a copper catalyst. Recovery of N from phenol/acetic acid/water extracts was about 30 per cent greater when sucrose (150 mg/ml of H_2SO_4) was added to the digests and this was done as a routine. Amino N was estimated colorimetrically using the reagent of Matheson *et al.*[32]

TCA extracts, when analysed for amino acids, were partitioned against ether and then dried *in vacuo* at 35°. The residue was dissolved in pH 2·25 buffer, 0·2 M, in Na^+, and amino acids separated by the method of Moore *et al.*,[33] using the temperature programme described by Oreskes *et al.*[34] to separate the amides, the 50 cm column to separate the basic amino acids other than arginine and the 15 cm column for arginine. Column effluents were monitored continuously using the Technicon Autoanalyser.

In fractionation experiments, extracts were prepared by grinding at 2° with sand in a mortar and pestle, and filtering through sintered-glass filters. Initial extraction was with 1 vol. of 10 per cent (w/v) TCA, and five extractions with 5 per cent TCA followed. The residue was rinsed with acetone, and extracted three times with $CHCl_3$/EtOH (2/1, v/v). The acetone rinse was bulked with the $CHCl_3$/EtOH extracts. These extracts were washed by the method of LeBaron and Folch.[35] Six extractions were made with phenol/acetic acid/water (1/1/1, w/v/v)

Preparation, Treatment and Ripening of Slices

Banana fruit slices (6–8 mm thick) were aseptically prepared and incubated as described elsewhere.[10] Inhibitors and metabolites were introduced into the slices by vacuum infiltration, using a variety of vacuum and time conditions which are detailed, where appropriate, in the text. In cell incorporation and uptake experiments, the peel, the placental zone and the outer 1–2 mm of pulp were removed immediately before extraction of the remaining central pulp tissue. Ethylene was applied either continuously in a stream of humidified air at 10–70 ppm, or over a 16 hr period at 20–60 ppm in a large sealed tank. Incubation was in the dark at 20°–25°. Ripening was assessed by independently scoring peel colour change, and the bending and swelling of the pulp tissue.[18] Each treatment score was the sum of both scores for five similarly treated slices, when each slice within a treatment was from a different fruit, and each fruit was represented in each treatment. Changes in soluble solid contents were estimated as described by Palmer and McGlasson.[10] Respiration measurements were made by the method of Claypool and Keefer.[36]

Radioactive Experiments

Generally, labelled L-^{14}C-lysine (189 μc/μmole) and L-^{14}C-valine (267 μc/μmole) were from The Radiochemical Centre, Amersham, Bucks., U.K. Radioactivity was usually measured in "Diotol"[37] scintillator. The fraction excluded from Sephadex G-75 gels equilibrated with PAW was accepted as protein. The solvent was removed *in vacuo* over H_2SO_4. The protein was dissolved in 0·2 N NaOH and measured by the method of Lowry *et al.*,[38] radioactivity was measured with the addition of 0·1 ml of formic acid per 16 ml of scintillator.[39]

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Valine and lysine were recovered as their dinitrophenyl derivatives,[40,41] and counted at infinite thinness using a Geiger–Müller thin end-window tube at an efficiency of about 8 per cent. Radioactive carbon dioxide was collected and measured by the method described by Godfrey and Snyder.[42]

To estimate the uptake of ^{14}C-amino acids by pulp cells, 1 mm thick slices were cut from the central pulp zone at appropriate times after infiltration. 5-g lots of these were bathed for 60 min at 2° in 100 ml of aerated distilled water. The diffusate was then decanted, and the tissue weighed. Assuming a tissue density of 1·0, the total ^{14}C which diffused was calculated as 105 × ^{14}C (cpm/ml) in the diffusate. Amino N diffusing was estimated similarly from the amino N concentration (μmoles/ml) in the diffusate. The diffused tissue was extracted at 2° in 10 per cent (w/v) TCA, and then five times in 5 per cent (w/v) TCA. Amino N and ^{14}C were measured in these extracts. The ^{14}C which diffused (*B* in Table 3) was expressed as a percentage of the ^{14}C recovered in diffusate and extract. The amino N which diffused (*A* in Tables 3, 4 and 5) was expressed as a percentage by a similar calculation.

If amino acids within cells did not diffuse from tissue slices into water, then TCA-soluble ^{14}C within the cells (C, Table 3), would equal $100 - B$. However, amino N, and likewise ^{14}C-amino acid from within the cells, does occur in the diffusate partly as a result of damage to cells in slicing and mostly due to cell leakage. This contribution was estimated by the factor $A/(100-A)$. Thus, TCA-soluble ^{14}C in the cells (C) was given by, $C = (100-B) + [A/(100-A)]\ (100-B)$. Total uptake into the cells (D, Tables 3, 4 and 5) included ^{14}C in PAW extracts also, and is expressed as a percentage of total ^{14}C recovered.

Gel Electrophoresis

Pulp samples which had been frozen and ground in liquid N_2, and stored at −80° were used. Extracts were made by grinding at 0° 5 g of tissue in 7 ml of freshly prepared 0·5 M $KHCO_3$ (pH 8·3), containing 1·0 per cent carbowax 6000, 0·1 per cent ascorbate and 5×10^{-4} M sodium mercaptobenzothiazole, using a nylon pestle in a polypropylene tube. Mercaptobenzothiazole is a potent inhibitor of enzymic browning.[43] The homogenate was centrifuged for 40 min at 40,000 *g* and the supernatant passed through a millipore filter (1·2 μ). Abour 50 per cent of the tissue protein was extracted by this means. Electrophoresis was commenced within 2 hr of starting the extraction.

Electrophoresis through polyacrylamide gel was by the method and in the buffer systems of Davis,[44] Ornstein,[45] Peacock *et al.*,[46] and Reisfeld *et al.*[47] Loadings, as measured by N precipitated in 5 per cent (w/v) TCA, were 170 μg for the anionic system and 105 μg for the cationic system. Protein zones were visualized with Amido Schwarz in 7 per cent (v/v) acetic acid, and excess dye was removed by diffusion. Gels were scanned with the Densicord recording electrophoresis densitometer, and were reproducible in different gels loaded with extracts from the same ripening stage.

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